Molecular Analysis of The First Reported Hereditary Lymphedema-Distichiasis Case in Bangladesh

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Research

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Abstract

Background

Lymphedema–distichiasis syndrome (LD, OMIM 153400) is a hereditary primary lymphedema with autosomal dominant nature of inheritance and variable expression. LD is characterized by late childhood or pubertal onset of lower limb lymphedema and an aberrant second row of eyelashes (distichiasis) arising from the meibomian glands. Underlying molecular causes include mutations in the *FOXC2* gene, which codes for a forkhead transcription factor involved in the development of the lymphatic and vascular system.

Results

In this study, we report the first case of LD from Bangladesh with classical lymphedema–distichiasis syndrome who carries an eight-base-pair deletion in the *FOXC2* -gene. ClinVar accession code for this deletion is RCV000007679.3. Although most other mutations of this gene are unique among different families, literature survey indicates this 8 bp deletion has been reported multiples times in independent studies for families from different geographical regions.

Conclusion

FOXC2 protein is 501 amino acid long. This deletion of 8 bp (ACGCCGCC) causes frameshift of codons after amino acid number 304. The frameshift creates an altered truncated protein with 154 newly amino acids after codon 304. We assume that these changes in the protein may affect its function contributing to the disease manifestations. Further research may confirm these assumptions.

Background

Lymphedema-distichiasis syndrome (LD) [MIM 153400]) is a form of hereditary lymphedema in which lymphedema, primarily of the limbs, with variable age at onset, is seen together with distichiasis, or double rows of eyelashes. The extra eyelashes grow from the meibomian glands and may protrude into the cornea, producing severe corneal abrasions. This combination was first described by Neel and Schull (1954) and by Falls and Kertesz (1964). Various additional complications such as cleft palate, cardiac defects, abnormal curvature of the spine, droopy eyelids etc (1) have also been observed.

The disease has been mapped to 16q24.3 (2) where the forkhead/winged helix transcription factor *FOXC2* is located in this region. Studies showed that dominant mutations in the *FOXC2* gene (MIM602402), cause lymphedema with variable age of onset (range: 7-40 years), often associated with distichiasis (3)(4). Key roles of FOXC2 include regulating differentiation of lymphatic endothelial cells, formation of smooth muscle cell layers and morphogenesis of lymphatic valves. Along with VEGFR-3, FOXC2 acts to establish distinct features of the lymphatic vascular architecture(5).

In both humans and mice, FOXC2 is highly expressed in the developing lymphatic vessels, as well as in the adult lymphatic valves(6). Its critical role in lymphatic vascular development has been discovered by the manifestation of abnormal lymphatic patterning and absence of proper lymphatic valves in Foxc2-deficient mice. LD patients develop similar defects characterized by lymph and venous reflux, indicating failure or absence of lymphatic and venous valves (7).

FOXC2 is transcribed to a 2.2 kb transcript containing a 1.5 kb single exon coding region. The FOXC2 protein contains 501 amino acids. The most characterized region in the gene is the fork-head DNA binding domain (FHD, amino acids 71 to 162). It also contains a nuclear localization signal (NLS1, amino acids 78-93). At the N-terminal there is a transactivation domain 1 (AD-1) starting from the first amino acid until the FHD (amino acid 71). In the C-terminal, a second transactivation domain (AD-2, amino acids 395-494) and an inhibitory region (ID-2, amino acids 495-501) have been identified (8). In the central region of FOXC2 protein, after the nuclear localization signal 2 (NLS2, amino acids 168-176), some phosphorylation and SUMOylation conserved sites have been recently identified (9)(10).

In LD patients, almost 70 different *FOXC2* mutations have been reported to date, scattered randomly along the whole coding sequence. The majority of *FOXC2* mutations are small insertions or deletions and nonsense mutations causing truncated proteins(11). It probably creates haploinsufficiency condition which explains the dominant nature of LD. The *FOXC2* haplo-insufficient state is associated to hyperplasia and distichiasis in mice (6). Mutations responsible for the disease greatly vary among affected families and the secondary phenotypes differ among families. None-the-less, any strong correlation has not yet been established between allelic variants and phenotypes. The disease exhibits variable penetrance of among the family members carrying the same allelic variant.

In this study, *FOXC2* gene of an individual diagnosed positive for Lymphedema distichiasis was analyzed by targeted Sanger Sequencing. The whole exon of 1501 bp was amplified through primer walking using five sets of primers. Due to high GC percentage of the gene, the primers were not efficient and PCR reaction mixture needed addition of certain additives and nested PCR was performed. It is the first molecular study of a Bangladeshi Lymphedema distichiasis patient and a homozygous 8 base pair deletion has been identified.

Subject And Methods

Subject and methods

The case

A 32-year-old normotensive, non-diabetic, male presented with a history of bilateral below knee swelling since the age of twelve. He also noted foreign body sensation in both eyes for the last 10 years and the recent appearance of whitish spots on the left eye with impairment of vision of the same eye for about a month. Patient’s father and one of his younger brothers are also suffering from the same kind of illness.

These characteristics indicated that the patient may be suffering from a certain type of hereditary lymphedema.

Mutational analysis of FOXC2 gene

Genomic DNA isolation

Genomic DNA was isolated from a 200 µl blood sample of the patient using GeneJet^{TM} DNA extraction kit following the standard isolation protocol. Concentration and purity of DNA was sufficiently good for PCR and sequencing.

DNA amplification: Due to absence of any reported mutational hot spot and presence of only one exon in this gene, it was planned to sequence the whole gene using a method called primer walking. The following 5 sets of primers were designed to amplify 5 products that overlap each other to cover the whole gene (Table: 1, Figure:5c).

All PCR reactions were performed using NEB PCR kit following standard reagent composition and reaction condition except template amount, annealing temperature and extension time. These were variable based on genomic DNA concentration, Tm of primer and product size of each PCR reaction. For retrieving full sequence, the following forward and reverse primer combinations were used: F1+R1, F2+R2, F3+R3, F4+R4, F5+R5, F1+R5, F2+R4. The annealing temperature of those combinations is 60^0C. PCR products were purified using ATP PCR/Gel DNA extraction kit following standard protocol.

Table 1
Primers for PCR amplification of FOXC2 gene

Primers	sequence	length	Tm	Size(bp)
F1	GAGCCGTCTCGGAAGCAG	18	60.66	401
R1	TCGTTGAGCGAGAGGTTGTG	20	60.32	
F2	TTCATCATGGACCGCTTCCC	20	60.11	524
R2	ATGTTCTCCACGCTGAAGCC	20	60.47	
F3	CAAGGAGGCCGAGAAGAAG	19	60.1	564
R3	GTGGTGCTGGTGGTGGTG	18	62.2	
F4	ATCATGACCCTGCGAACG	18	60.7	598
R4	TGCCACTCACCTGGGACT	18	60.3	
F5	GCCTCCTGGTATCTCAACCA	20	60.1	328
R5	TCTCTGCAGCCCCTTAATTG	20	60.3	

Sanger sequencing of PCR products: The purified DNA was used as a template for the cycle sequencing reaction with the Big Dye Terminatorv1.1 Cycle Sequencing Kit with different forward or reverse primer of the respective product. Conditions for this cycle PCR on thermo cycler were: 1 minute for an initial denaturation of the DNA at 96^0C, followed by 35 cycles of a 10-second denaturation at 96^0C, variable annealing temperature based on the primers used for 5 seconds, and the extension step at 60^0C for 4 minutes. After this, capillary electrophoresis was performed on the 3130 Genetic Analyzer.

Results

Clinical features and diagnosis

The patient was admitted with characteristics indicating certain type of hereditary lymphedema. All the parameters of general examination including the vitals were normal. Examination of lower limbs revealed bilateral edema of legs and feet with dryness and hyperpigmentation of the overlying skin. Examination of the eye revealed partial ptosis on the left side, extra partial set of eyelashes on both eyes and left corneal opacity with reduced visual acuity on left eye. Slit lamp examination of both eyes showed bilateral distichiasis, epithelial and stromal opacity in the center of cornea and superficial corneal vascularization on the left eye. Results of other systemic examinations were normal. Isotope Lymphoscintigraphy of both lower limbs revealed grade-1 and grade-2 lymphoedema on the left and right side respectively. Findings of serum creatinine, ALT, Prothrombin time, urine R/M/E, ECG, Echocardiogram and CXR were normal. ICT for filaria was also negative. Duplex study of both lower limbs found no evidence of deep vein thrombosis and no evidence of arterial insufficiency.

Based on these clinical features and the patient statement of his father and younger brother having similar illness, he was diagnosed with a rare hereditary disease, Lymphedema distichiasis syndrome.

After necessary documentation, blood sample from the patient was collected to test for the presence of *FOXC2* mutations.

PCR results of *FOXC2* gene

PCR amplification of the 5 primer sets showed specific bands only for pair number 1 and 5 (Figure: 3a). Regions covered by primer pair one and five were sequenced (Figure:4).

To reveal the sequence between, several modifications were tried. Interestingly, gel electrophoresis indicated amplification from primer combination of F1 and R5 with 5% DMSO (Figure:3b). Annealing and extension temperatures were 60^0C and 72^0C respectively for this reaction. Later, this product was used as template to perform nested PCR with primer combination of F2 and R4. In this nested PCR reaction mix, annealing temperature was 60^0C and 3% DMSO was added considering presence of DMSO in the product to be used as template. Nested PCR with primer combination of F2 and R4 resulted in better amplification but nonspecific products were present (Figure: 3c).

The band of desired size of 1120 bp was isolated using Gel extraction after electrophoresis. This 1120 bp DNA was sequenced using Sanger method from both primers. PCR products of primer pair F1+R1, F2+R4 and pair F5+R5 overlap each other and cover the whole coding region of *FOXC2* gene. So, these three products were sequenced to get the complete sequence of the gene (Figure:4).

Sequence analysis of FOXC2 gene: Primer pairs of PCR products that provided good quality sequence are F1+R1, F5+R5 and F2+R4. When blast search against the NCBI nucleotide database were performed, no mutation was found in the sequence derived from pair F1+R1 and F5+R5. Product of primer combination F2+R4 was sequenced with both F2 and R4 primer to get the sequence of the whole segment. Sequence from R4 was reverse complemented and after trimming they were assembled in full 1120 bp sequence. When the assembled sequence was blast searched, a 8 bp deletion (ACGCCGCC) was identified (Figure:5a). The chromatogram also indicates that the patient is homozygous for this deletion (Figure:5b). This deletion is identified to be between base no 6423 and 6431 of RefSeqGene on chromosome 16, (which refers to mRNA position between 914 and 921). This mutation had been reported before for the same disease. ClinVar accession code for this deletion is **RCV000007679.3**. Although most other mutations of this gene are unique among different families, as of October, 2021 only this 8 bp deletion has been reported four times in four independent studies for families from different geographical region (4,11–13). These changes resulted in the production of a premature stop codon that terminated the predicted protein earlier than the wild-type and produced novel C-termini. Codons after 304 are disrupted for this mutation and new 154 amino acids are added.

Discussion

Although Hereditary lymphedemas are rare, analysis and investigation of such disease bear significance. The *FOXC2* gene involved in the lymphedema distichiasis disease mentioned in this study, has been found out to be an important marker for cancer prognosis and metastasis.

Most frequent FOXC2 mutations are frameshift in nature(1,4). Most take place 3' to the fork-head-specifying region of the gene, suggesting that putative mutant proteins would retains the activity of this essential DNA-binding domain. In contrast to most other mutations, which appear to be unique, the eight-base-pair deletion reported in this study was observed in four additional unrelated pedigrees with lymphedema and distichiasis, suggesting that it is recurrent. One study reported this deletion in a French family showing autosomal-dominant segregation of upper- and lower-eyelid distichiasis in seven affected relatives over three generations, in addition to below-knee lymphedema of pubertal onset in three(12). Two children had cleft palate as well as distichiasis, but without any association with the Pierre–Robin sequence. Divergent strabismus and early-onset myopia were other ophthalmologic anomalies. Another study reported this deletion in a British family with the father and all three of his children being affected with both distichiasis and bellow-knee lymphedema(4). Another study reported this mutation in fourteen-year-old boy with distichiasis, cleft palate webbed neck and very early onset (6 week) lymphedema(13). The patient of this study stated he developed lymphedema during his puberty. He had distichiasis in the lower lids of his both eyes. He did not have myopia or cleft palate like some

other LD patients with this deletion. Being reported in this study makes this 8 bp deletion the only *FOXC2* mutation that has been found in five totally unrelated LD affected families. The reason behind the recurrence of this 8-base-pair-deletion is not fully clear. It indicates that the region is prone to DNA polymerase slippage during replication process. In fact, high GC percentage has been reported to make DNA more prone to deletion through polymerase slippage and recombination which partly explains why most of the *FOXC2* mutations are frameshift deletions. Elucidating the reason behind this particular deletion being relatively more frequent might shed light on to important features that make a sequence prone to deletion and has implications in mutational hotspot identification.

The patient diagnosed in this study draws special attention due to relatively low expressivity of the disease even though he is homozygous for the deletion in *FOXC2*. The deletion disrupts codons after 304. It means that C-terminal activation domain and inhibitory domain are disrupted but the amino acid sequence for N-terminal activation domain, nuclear localizing segment and forkhead DNA binding domain remains identical to native protein. As the homozygous deletion did not result in serious health complication for the patient, the mutated protein probably retains most of its native functions. Although proper function of the protein might depend on the interactions among its domains, function of the N terminal activation domain and forkhead DNA binding domain was not fully disrupted otherwise no native function would have been retained. A study found eight proline-directed Ser/Thr phosphorylation sites in FOXC2 clustered in a relatively short region of the protein encompassing amino acids 219 to 367. Phosphorylation at these sites was found to be important for FOXC2-induced vascular remodeling in vivo. It indicates that this eight-base-pair deletion probably hampers the proper regulation of the protein but its major functional domains maintain their functions.

Unfortunately, the sequence of a 40 bp segment could not be clearly read from the chromatograms due to the limitation of Sanger sequencing to produce peaks of good resolution at the ends of the DNA molecule (Figure:5c). Extensive literature search has ensured that there is no report of mutation in this region and no LD patients were found to simultaneously carry two different mutations of *FOXC2* gene until now.

Conclusion

This is the first reported case of LD in Bangladesh. The frameshift mutations in *FOXC2* in this patient and the other mutations reported previously produce premature stop codons with varying lengths of novel codons between the mutation site and new C-terminus. It is noteworthy that many of those mutations would add a long stretch of novel amino acids. Deleterious mutations of *FOXC2* in this disease follow dominant nature of inheritance. In a large-enough-family symptoms of variable severity, onset and spectrum are observed. This variable expressivity and frequently occurring truncating mutations strongly suggest haploinsufficiency as underlying mechanism. In some cases, addition of a large novel polypeptide shows no phenotypic difference from the single point mutation.

Declarations

Ethics approval and consent to participate: N/A

Consent for publication: Attached

Availability of data and materials: All data are included in this manuscript.

Competing interests: The authors declare no competing interests

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Authors' contributions: The clinical assessments were conducted by Md. Lutfar Rahman, Md. Mostafizur Rahman, Mohammad Ali and A.F.M Helal Uddin. The molecular study was planned and supervised by Dr. Mustak Ibn Ayub and bench work was conducted by Nahid Parvez.

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Figures

Figure 1

Structural domains of FOXC2 protein: transactivation domain 1 (AD-1), fork-head DNA binding domain (FHD), nuclear localization signal 1(NLS1), nuclear localization signal 2 (NLS2), transactivation domain 2 (AD-2), inhibitory region 2 (ID-2).

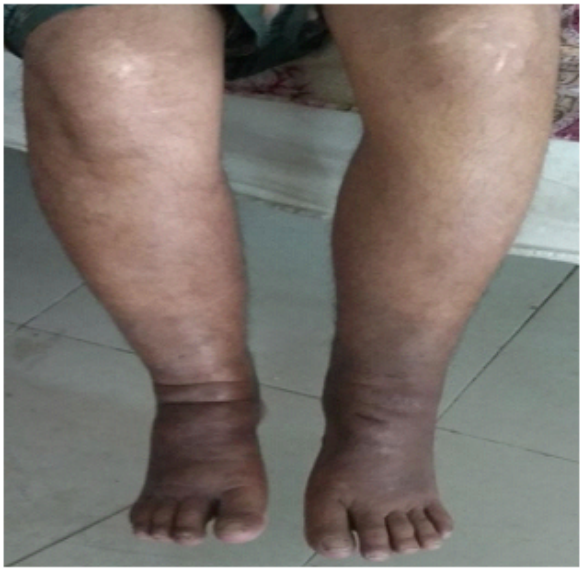


Figure 2a

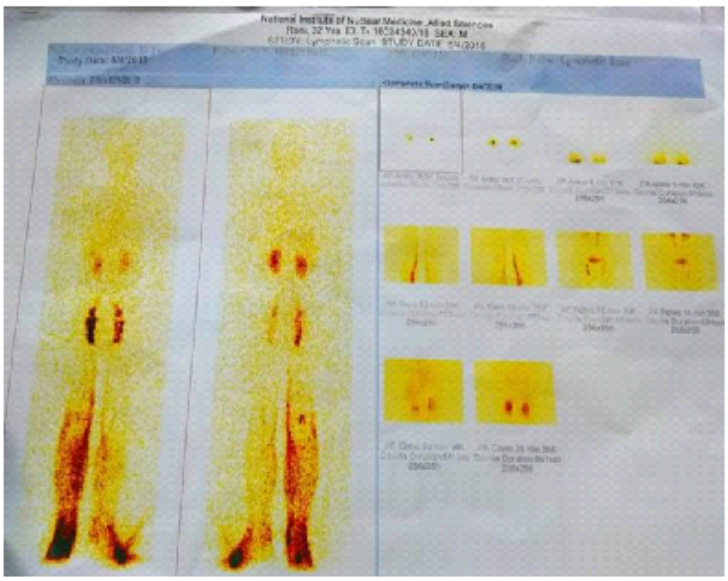


Figure 2b

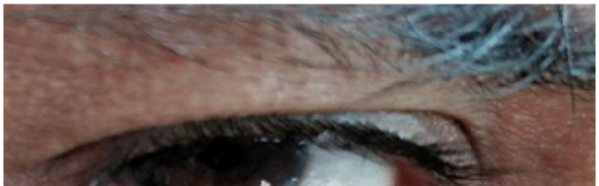


Figure 2c (Right eye)

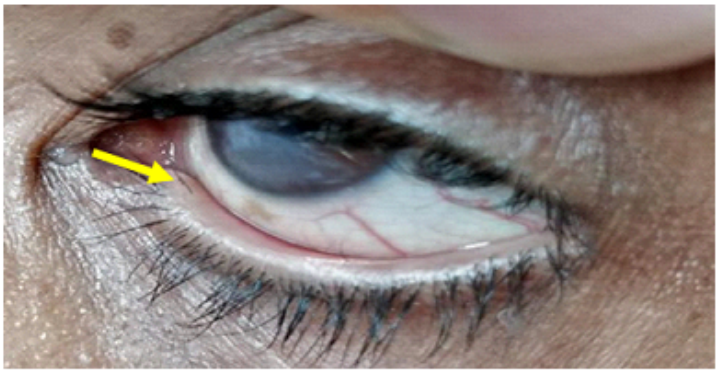


Figure 2d (left eye)

Figure 2

clinical presentation of a 32-year-old Bangladeshi male with lymphedema distichiasis, (a) the lymphedema of the legs and feet, (b) Lymphoscintigraphy image of both legs, (c,d) Distichiasis in lower eye lids

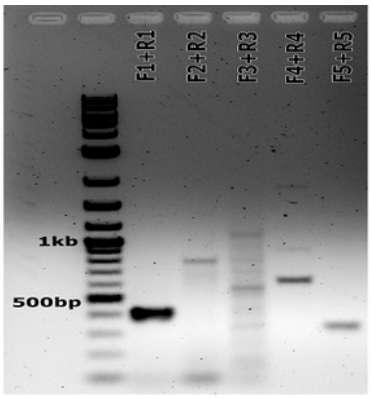


Figure 3a

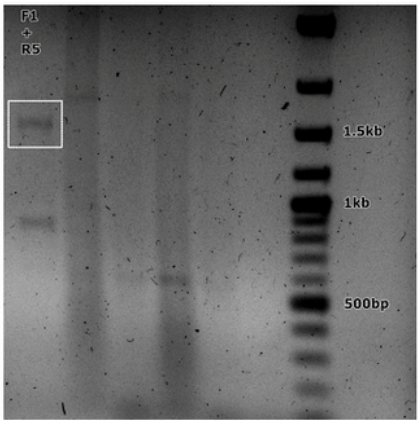


Figure 3b

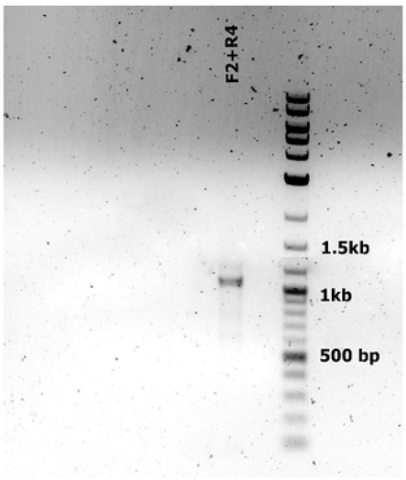


Figure 3c

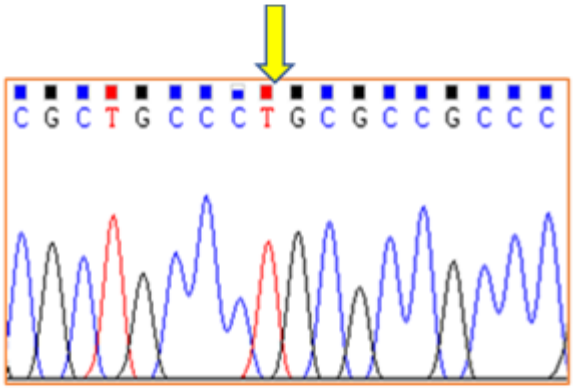
Figure 3

(a) PCR amplification with all FOXC2 primer pairs. Among these, good quality sequence was determined from pair 1 and pair 5; (b) Gel electrophoresis of PCR amplification with DMSO, band was visible only for primer pair: F1+R5. The band of 1.5 kb size (indicated by the white box) was extracted from gel to use as template for nested PCR; (c) Nested PCR with product of F1+R5 as template, PCR product of primer F2+R4 was loaded in the gel and band of 1120 bp is visible with slight nonspecific amplification.

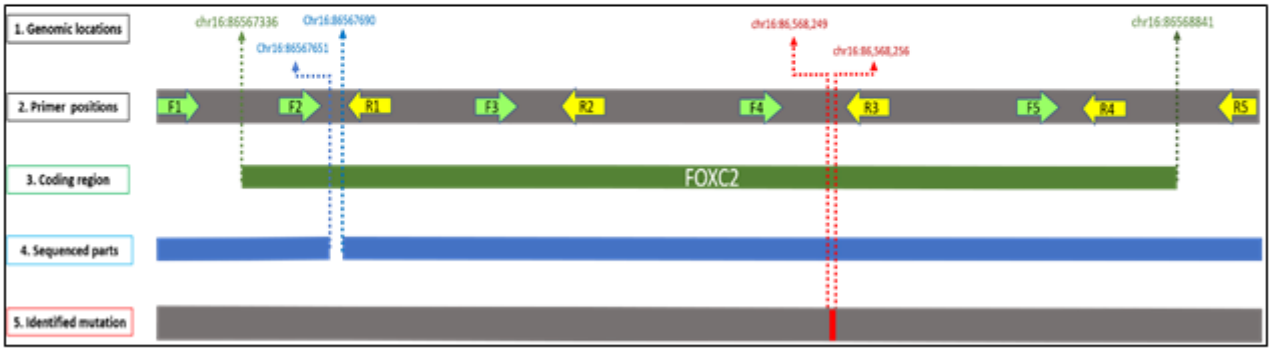
Figure 4

PCR amplification maps of the FOX2 gene. F:forward primer, R: reverse primer, numbers after F/R means pair number, green bars indicate sequenced region, pink arrows indicate primers used during cycle sequencing.

5a



5b



5c

Figure 5

(a) Blast result showing 8-base pair deletion, (b) Chromatogram of patient's DNA, yellow arrow indicates site of mutation. (c) relative position of primer pairs [2] and genomic locations of FOXC2 coding region [3], sequence gap [4] and identified mutation [5]; genomic locations are given according to GRCh38.